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INTRODUCTION

Our overall objective is to identify molecular elements that enable breast cancer cells to establish metastases. Finding targeted approaches to inhibit rate-limiting events of metastatic growth is preferable to using therapeutics that are cytotoxic on a systemic level. Cadherins make up a family of adhesion molecules that mediate Ca2+-dependent cell-cell adhesion at points of cell-cell adhesion (Goodwin and Yap, 2004). Epithelial-cadherin (E-cadherin), the prototype classical cadherin present on the surface of most epithelial cells, has a cytoplasmic domain that anchors the cell adhesion molecule to the actin cytoskeleton via catenin-based complexes. It is generally considered that E-cadherin directs homotypic binding, organizing cells of the same lineage into a functional tissue during morphogenesis (Pla et al., 2001). Thus, E-cadherin is central to epithelial cell differentiation and suppression of proliferation and migration.

Finding E-cadherin downregulated or even lost in invasive and metastatic carcinomas buttressed this role of E-cadherin in modulating the epithelial phenotype (Hirohashi, 1998). It has been hypothesized that loss of E-cadherin allows individual tumor cells to break from the primary tumor mass at the same time as enabling autocrine pro-proliferative and –migratory signaling to ensue from receptors and ligands physiologically separated by cell polarity and the E-cadherin-based tight junctions. This was supported experimentally when poorly differentiated and invasive carcinoma cells could be made less so by transfection with E-cadherin cDNA, with well-differentiated carcinomas becoming more aggressive when antibodies blocked. This supported a designation as a tumor suppressor, even placing E-cadherin at the apex of a "tumor suppressor system" (Vleminckx et al., 1991). More recent reports of E-cadherin being expressed at the site of metastatic foci in the liver, lung and lymph nodes (Kowalski et al., 2003) have caused reconsideration of E-cadherin downregulation as required for tumor dissemination. The key question is whether downregulation of E-cadherin is not required for dissemination, or rather, as we posit here, that E-cadherin expression and functionality is re-established at the metastatic site. *Our central hypothesis tests whether E-cadherin is necessary for cohesion between invasive breast cancer cells and the target hepatic parenchyma and that the formation of E-cadherin and connexin foci is a major rate limiting step in establishing metastatic disease.* In our first 12 months of DoD funding, we have made considerable strides into our objective of determining the importance of E-cadherin in the context of metastatic cancer cells in a hepatocyte microenvironment.

BODY

The Statement of Work (Table 1) described two tasks to effectively test our hypothesis. We have tackled the tasks in the order of greatest important, while keeping to the schedule set forth in the original proposal. The central effort in this first year of funding has been focused on establishing the nature of the E-cadherin interaction between invasive breast cancer cells and hepatocytes.

Table 1. Statement of Work

Task 1A. examine the single cell architecture of breast cancer cells interactions with hepatocytes by microscopy – *completed*
Task 1B. determine the strength of the interactions using a centrifugal assay – *completed*

Task 2A. monitor protein localization using fluorescently-tagged E-cadherin – *completed (attempted real-time localization but not successful; evaluated by fluorescent microscopy)*
Task 2B. probe connexin unit integrity and transference – *completed*

Task 2C. assess the organ relevance of the cohesive interactions using an ex vivo liver bioreactor system – *completed*

Task 1.A. *Examine the single-cell architecture of breast cancer cell's interaction with hepatocytes by microscopy.* This task is completed. We have been successful in capturing the interaction between breast cancer cells and hepatocytes. Using human MCF7 breast cancer cells and freshly isolated rat hepatocytes, we co-cultured the cells together for 90 minutes. We observed that actin localizes to points of juxtaposition between breast cancer cells and hepatocytes (Figure 1A); further, we observed that Arp2/3, the best understood molecular determinant for actin polymerization, co-localizes with E-cadherin plaques directly juxtaposed to hepatocytes (Figure 1B). Together, these data suggest that breast cancer cells are actively anchoring themselves to hepatocytes via E-cadherin.

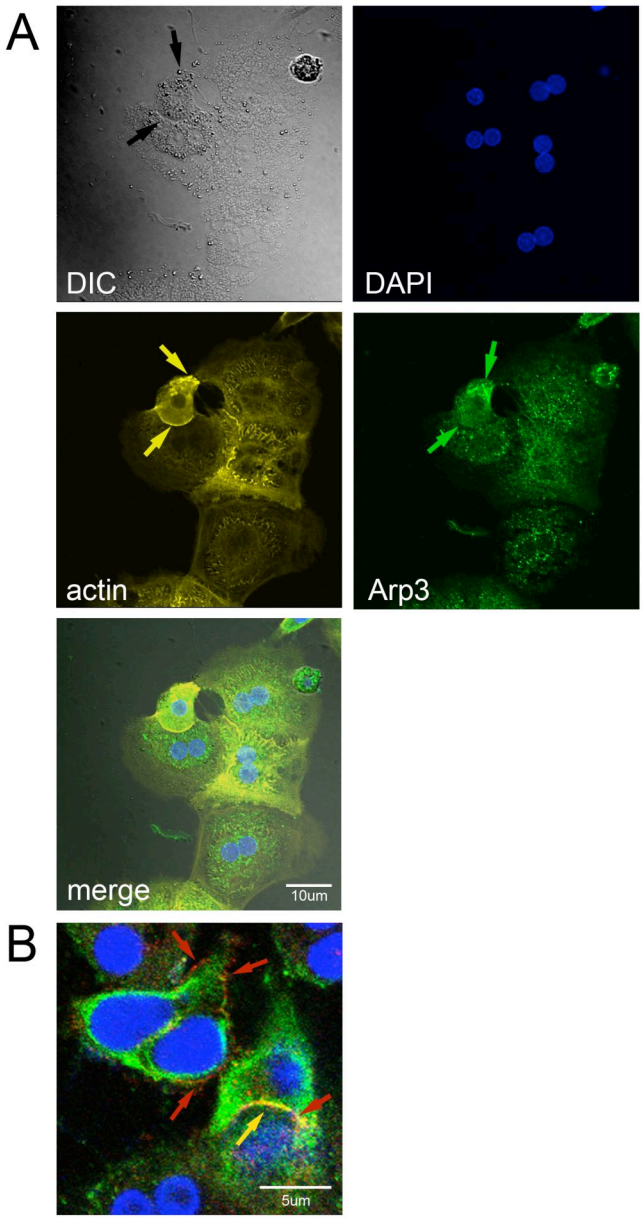

Figure 1. (A) The DIC frame shows a breast cancer cell interacting with hepatocytes. The well-differentiated multi-nucelated hepatocytes can be distinguished from the mono-nucleated cancer cell in the DAPI inset. Actin co-localizes with Arp3 at the juxtaposed membranes. (B) Arp3 also co-localizes with E-cadherin plaques on the membranes of breast cancer cells interacting with rat hepatocytes. Human-specific E-cadherin antibody (red), pan-species Arp3 antibody (green).

Task 1.B. *Determine the strength of the interactions using a centrifugal assay*. This task is completed. During this first year, we were able to optimize a centrifugal assay to study the adhesion between breast cancer cells and hepatocytes. We found that E-cadherin positive MCF7 breast cancer cells are able to form stable adhesions with hepatocytes in a similar manner to their ability to form stable adhesions with themselves. E-cadherin-negative MDA-231 cells do not form stable adhesions with hepatocytes. Further, if we disable the E-cadherin adhesion mechanism using calcium chelation or a function blocking antibody, we are able to abrogate the cohesion of the MCF7 cells to near background levels. An siRNA construct directed to E-cadherin, which knocks-down the protein significantly, also abrogates cohesion with the hepatocytes (see Figure 2).

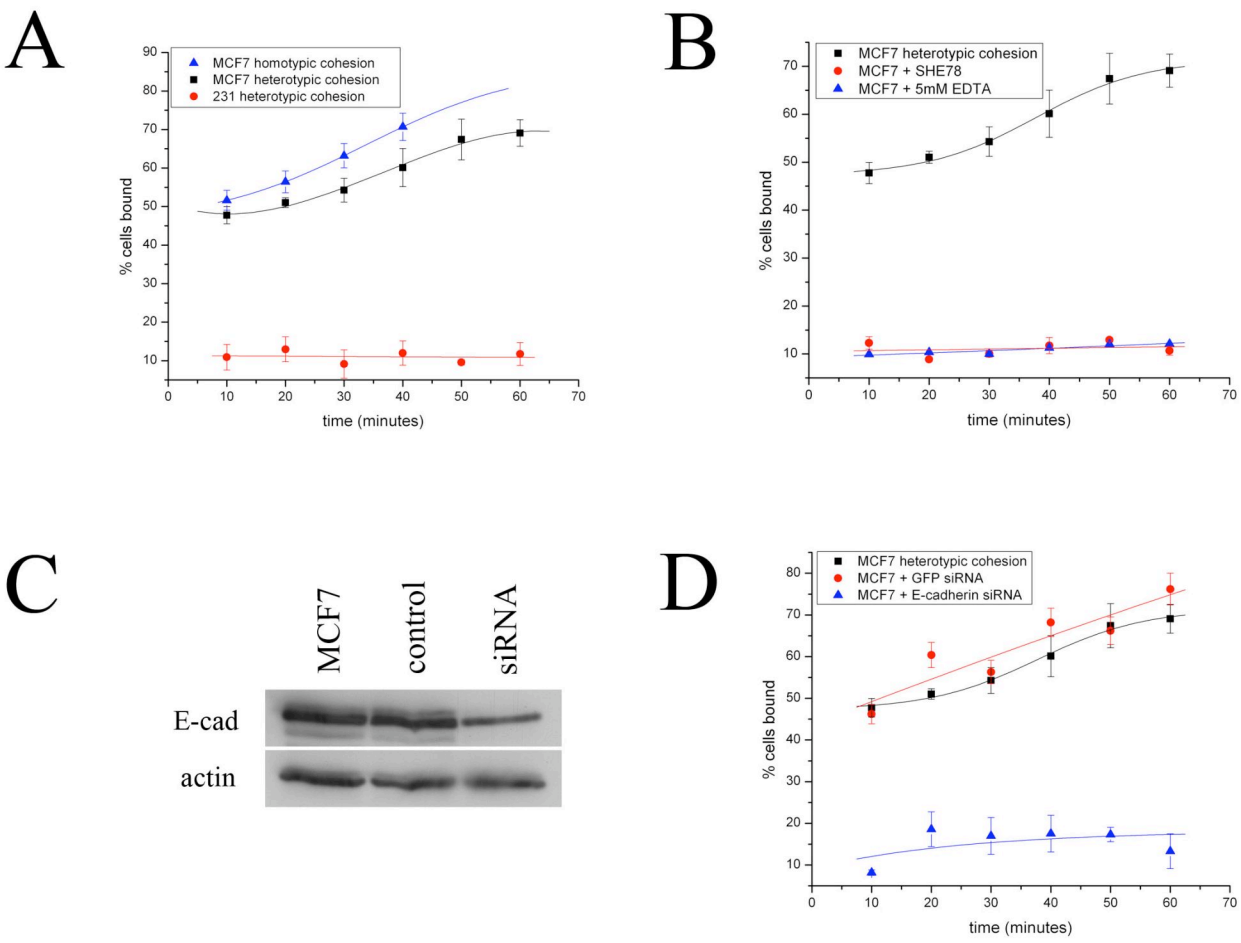

Figure 2. (A) Homotypic cohesion between MCF7-MCF7 populations occurs very similarly to heterotypic cohesion between MCF7-hepatocyte populations. MDA-231 cells do not effectively adhere to the hepatocyte population. (B) Use of calcium chelation or an E-cadherin function blocking antibody abrogates cohesion to near background levels. (C) An siRNA E-cadherin construct knocks-down E-cadherin in MCF7 to <30% of endogenous levels. (D) MCF7 cells transfected with E-cadherin siRNA adhere minimally to the hepatocyte population, while the siRNA control cells adhere similarly with the untreated MCF7 cells.

Task 2.A. *Monitor protein localization using fluorescently-tagged E-cadherin.* This task is in progress. We found that when E-cadherin-negative MDA-MB-231 cells were cultured with hepatocytes (described in supplemental data), the breast cancer cells re-expressed E-cadherin and appeared to interact with hepatocytes. To further characterize this interaction, we used immunofluorescence to localize the expression of E-cadherin. MDA-MB-231 cells cultured with hepatocytes appear to ligate with hepatocytes (Figure 3B) although due to autofluorescence of the hepatocytes and breast cancer cells it was difficult to ascertain whether there was expression of E-cadherin. As a result, we repeated these experiments with RFP-labeled MDA-MB-231 cells to more easily distinguish breast cancer cells from heaptocytes. Using this method, we were able to detect MDA-MB-231 cells that also stained for E-cadherin. However, we were unable to determine whether E-cadherin was located at the membrane. Additional studies are necessary to evaluate the hepatocyte-breast cancer cell interface at higher magnification to localize E-cadherin.

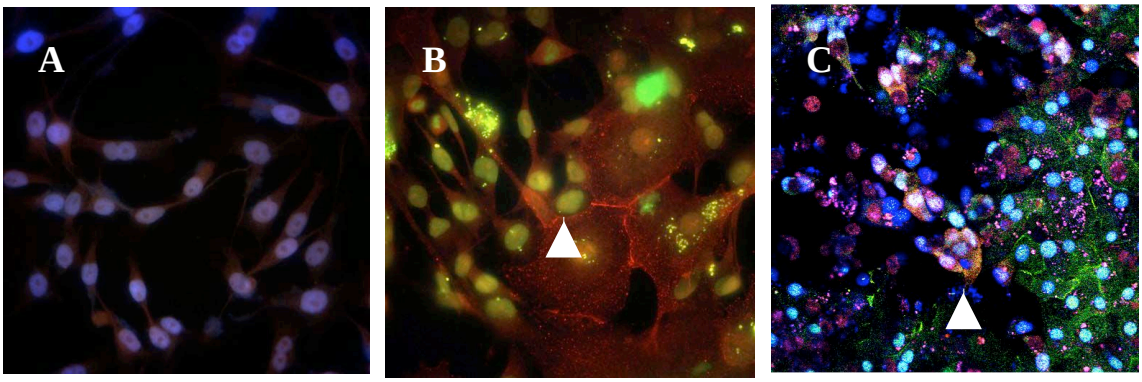

Figure 3. (A) MDA-MB-231 cells cultured in the absence of hepatocytes (B) MDA-MB-231 cells cocultured with hepatocytes for 10 days. Cells were stained with DAPI (blue), E-cadherin (red), and Hep Par 1 (green). (C) MDA-MB-231-RFP cells were cocultured with hepatocytes for 10 days. Cells were then stained with DAPI (blue) and E-cadherin (green). Arrowhead indicates MDA-MB-231-RFP cells also staining for E-cadherin.

Task 2B. *Probe connexin unit integrity and transference.* This work is completed. Gap junctions are involved in cell-cell communications and are comprised of connexin molecules. Connexins are often downregulated in breast cancer and have been shown to be tumor suppressors (McLachlan et al., 2006). We repeated the coculture experiments developed in Task 2A and analyzed the effect of hepatocyte coculture on Connexin43 expression. MDA-MB-231 cells normally do not express Connexin43. However, after 10 days of coculture with hepatocytes, punctate molecules of Connexin43 were visualized by immunofluorescence (Figure 4C). In addition, these molecules appear to be polarized in the direction of the hepatocytes, suggesting that MDA-MB-231 cells are establishing intercellular communications.

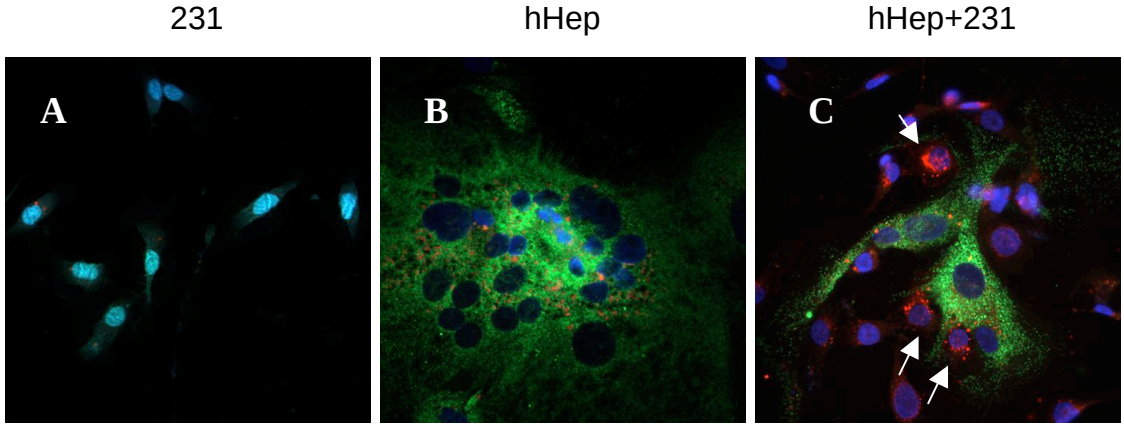

Figure 4. (A) MDA-MB-231 cells cultured in the absence of hepatocytes (B) Hepatocytes cultured in the absence of MDA-MB-231 cells (C) MDA-MB-231 cells cultured with hepatocytes for 10 days. Cells were immunostained for Connexin43 (red), hepatocyte marker Hep Par 1 (green), and DAPI (blue).

Task 2.C. *Assess the organ relevance of the cohesive interactions using an ex vivo liver bioreactor system.* This task is completed. Analysis of breast cancer cell and hepatocyte interactions in a liver bioreactor have many advantages over a 2D culture system. 3D bioreactors more accurately recapitulate many aspects of the liver microenvironment, including fluid dynamics, gene and protein expression, and metabolism. We therefore used this model to determine whether breast cancer cells form cohesive interactions with hepatocytes *ex vivo*. RFP-labeled MDA-MB-231 cells were seeded into the liver bioreactor and cultured for 10 to 15 days. Cells were then immunostained for E-cadherin and imaged by confocal microscopy. Similar to what was observed in Figure 3C, MDA-MB-231-RFP cells stained positive for E-cadherin following culture in the liver bioreactor (Figure 5). These results indicate that breast cancer cells are able to cohere to hepatocytes in both 2D and 3D environments. However, we were again unable to determine the localization of E-cadherin.

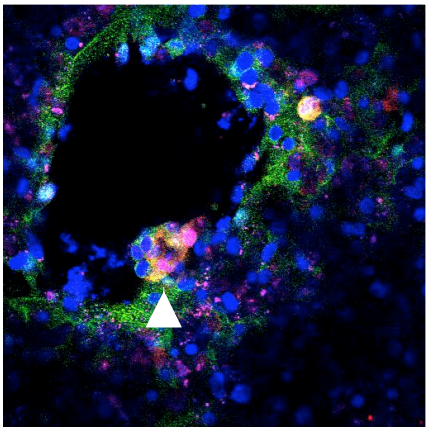

Figure 5. Hepatocytes were seeded into a liver bioreactor and allowed to organize on the collagen-coated scaffolds for 48 hours. Then RFP-labeled MDA-MB-231 cells were seeded into the liver bioreactor and cultured for 10 days. Scaffolds were stained for E-cadherin (green) and DAPI (blue). MDA-MB-231-RFP cells re-expressing E-cadherin (yellow) indicated by arrowhead.

Supplemental Data:

Determine whether E-cadherin binding between breast cancer cells and hepatocytes initiate survival signals in the tumor cells.

During the course of the experiments, it became obvious that upon E-cadherin ligation between cancer cells and hepatocytes that canonical pathway signaling could occur. This unexpected tumor cell interaction with its metastatic micro-environment is postulated to underlie the phenomenon of chemo-resistance of breast cancer mestastases even when the primary lesion responds to chemotherapy. We examined whether breast carcinoma cell interactions with hepatocytes elicited the canonical survival pathways (ERK MAP kinase and Akt/PKB) in the breast cancer cells. Indeed, these connections activated a sustained Erk-MAPK and Akt/PKB signal (Fiugure 6).

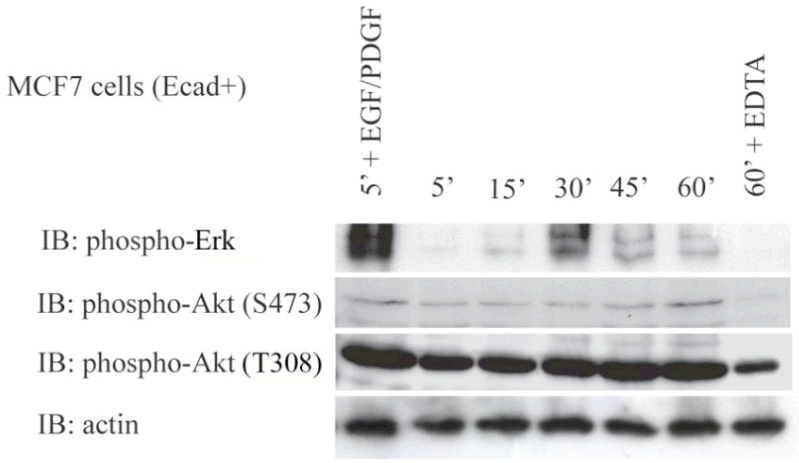

Figure 6. The Erk-MAPK and Akt pathways are activated in E-cadherin positive MCF7 cells upon ligation with hepatocyte E-cadherin. E-cadherin positive MCF7 breast cancer cells were seeded onto culture plates decorated with hepatocyte membranes. Erk activation peaks at 30' after ligation and Akt activation peaks at 60' after contact; activation of Erk and Akt can be attenuated with calcium chelation..

Determine whether E-cadherin negative invasive cancer cells re-express E-cadherin upon coculture with hepatocytes.

All of the experiments proposed in these studies used E-cadherin positive breast cancer cells (the MCF7 cell line). In order for our hypothesis to be relevant in the setting of metastatic pathogenesis, it was necessary to determine whether cancer cells that had lost E-cadherin due to methylation of the promoter region were able to convert and re-express E-cadherin upon coculture in the hepatic microenvironment. Using two DNA methylation-silenced breast cancer cell lines, MDA-MB-231 and MDA-MB-435, we observed that upon coculture with hepatocytes, the invasive cancer cells re-expressed E-cadherin and were thus able to participate in the adhesion and signaling activities shown above (Figure 7).

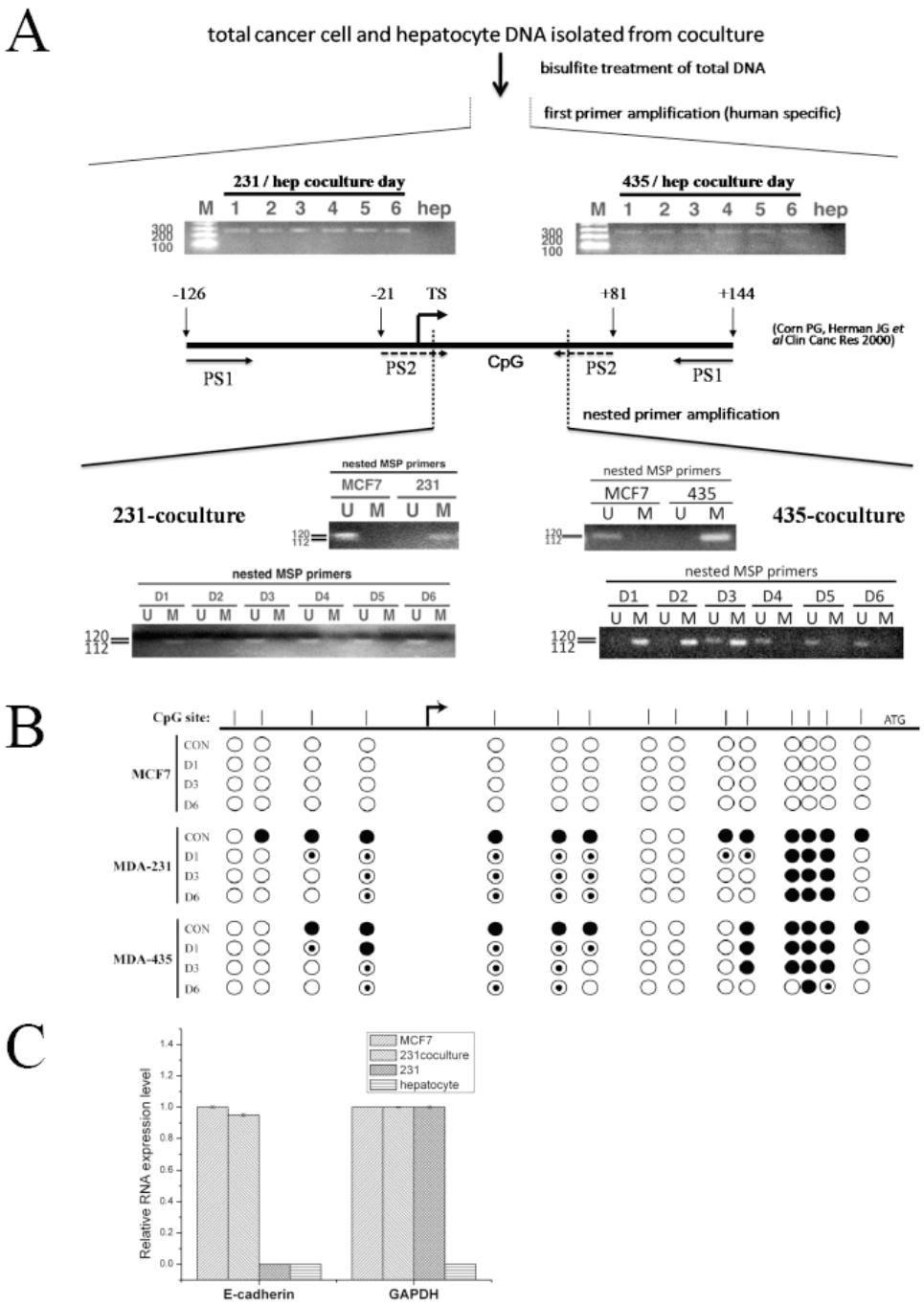

Figure 4. (A) Nested PCR method to detect methylation status of the E-cadherin promoter in a six day time course of hepatocye coculture. Above, bisulfite-treated DNA is amplified with primers that exclude CpG islands to amplify a 270bp region independent of methylation status. Below, nested primers anneal to the 270bp target to amplify a methylated (112bp) or unmethylated (120bp) fragment in the six day time course. MCF7 is used a unmethylated control. (B) Bisulfite sequencing of CpG islands in the E-cadherin promoter. Figure adapted from Corn *et al* (Corn et al., 2000). CpG islands are indicated as vertical lines on map; each CpG island is represented a circle. MCF7, MDA-MB-231, and MDA-MB-435 were sequenced on days 1,3, and 5 coculture. Open circle, unmethylated CpG; closed circle, methylated CpG; filled circle, mixed quality values. (C) RT-PCR using human-specific primers of MDA-MB-231 cells after 6 days of co-culture with hepatocytes. Means(n=4)±s.d. Note that species specific primers do not amplify E-cadherin or GAPDH from hepatocytes.

KEY RESEARCH ACCOMPLISHMENTS

1. MCF7 cells localize actin, which functions as a cytoskeletal anchor for cell adhesion molecules, to points of juxtamembrane contact with hepatocytes.
2. MCF7 cells co-localize Arp2/3 and E-cadherin to points of juxtamembrane contact with hepatocytes.
3. Heterotypic binding between MCF7 cells and hepatocytes occurs in a single logarithmic step with kinetics similar to homotypic binding of MCF7 cells.
4. Functional heterotypic binding between MCF7 cell and hepatocytes is E-cadherin dependent and can be abrogated using calcium chelation, function blocking antibodies, and siRNA specific to E-cadherin.
5. Canonical pathway signaling occurs when E-cadherin cohesion between heptatocytes and cancer cells is initiated.
6. E-cadherin re-expression was observed in invasive breast cancer lines whose E-cadherin was epigentically silenced by promoter DNA methylation, thereby making these key research accomplishment relevant to the pathogenesis of metastatic cancer.
7. E-cadherin re-expression occurs in 2D and 3D hepatocyte coculture, suggesting parallels to breast cancer metastatic to the liver.
8. Coculture with hepatocytes also results in re-expression of Connexin43 in MDA-MB-231 cells, suggesting that other mechanisms of cellular communication besides E-cadherin are being initiated between breast cancer cells and hepatocytes.

REPORTABLE OUTCOMES FOR YEAR 3

Abstracts:

Chao Y and Wells, A. “Re-expression of E-cadherin on metastatic breast cancer cells in the liver microenvironment.” National MD/PhD Meeting. Keystone, CO. July 2009.

Chao Y and Wells A. “Re-expression of E-cadherin on metastatic breast cancer cells in the liver microenvironment.” **Podium** American Association for Cancer Research. Denver, CO. April 2009.

Chao Y, Shepard CR, Wells, A. “E-cadherin as a chemotherapy resistance mechanism for breast cancer metastasis.” **Winner, student posters** University of Pittsburgh Science Symposium. Pittsburgh, PA. October 2008.

Chao Y, Shepard CR, Wells, A. “E-cadherin as a chemotherapy resistance mechanism for breast cancer metastasis.” **Podium** Academy of Clinical Laboratory Physicians and Scientists. Philadelphia, PA. May 2008.

Chao Y, Shepard CR, Wells, A. “E-cadherin as a chemotherapy resistance mechanism for breast cancer metastasis.” DOD Breast Cancer Research Program Era of Hope meeting. Baltimore, MD. June 2008.

Papers:

Chao Y, Shepard CR, Wells, A. “E-cadherin contributes to the mesenchymal to epithelial reverting transition”. *In preparation.*

PRIOR YEARS REPORTABLE OUTCOMES

Abstracts:

Shepard CR, A Wells. E-cadherin re-expression in breast cancer cells as a putative marker for tumor cell dormancy modeled by infiltration into hepatocyte spheroids. **Podium**; Cancer Epigenetics. Experimental Biology. San Diego, CA. 2008.

Shepard CR, A Wells. Demethylation of the E-cadherin promoter driven by hepatocytes allows of cell fate-determining signals in invasive breast cancer cells. **Podium**; Understanding Cancer for Improved Prognosis: Advances in Tumor Biology. Experimental Biology. Washington, DC. 2007.

Shepard CR, A Wells. Demethylation of the E-cadherin promoter driven by hepatocytes allows of cell fate-determining signals in invasive breast cancer cells. **Podium**; Highlights: Graduate Student Research in Pathology. Experimental Biology. Washington, DC. 2007.

Shepard CR, A Wells. Re-expression of E-cadherin by invasive breast cancer cells as a strategy for metastatic colonization of the liver. **General Session Podium**. San Antonio Breast Cancer Syposium. San Antonio, TX. 2006

Shepard CR, A Wells. Re-expression of E-cadherin by invasive breast cancer cells as a strategy for metastatic colonization of the liver. **Podium**. Biological Science Graduate Student Association Symposium. University of Pittsburgh School of Medicine. Pittsburgh, PA. 2006.

Shepard CR, A Wells. Cadherin interaction as a pathological adhesion mechanism in metastatic breast cancer. Abstract. Gordon Conference: Cell Contact and Adhesion. Andover, NH. 2005.

Reviews:

Wells A, C Yates, **CR Shepard** (2008). Mesenchymal to epithelial reverting transitions during the metastatic seeding of disseminated carcinomas. Clin Exp Metastasis, in press.

Papers:

Yates C, **CR Shepard**, G Papworth, A Dash, DB Stolz, S Tannenbaum, L Griffith, A Wells (2007). Novel three-dimensional organotypic liver bioreactor to directly visualize early events in metastatic progression. Adv Cancer Res *97*, 225-246.

Yates CC, **CR Shepard**, D Stolz, A Wells (2007). Co-culturing human prostate carcinoma cells with hepatocytes leads to increased expression of E-cadherin. Br J Cancer *96*, 1246-1252.

CONCLUSIONS

With the conclusion of the third year of the award, all of the tasks have been completed. The first part of the hypothesis has been repeatedly supported in the first task of the proposal. This study has also highlighted new directions concerning the signaling mechanisms that may be propogated upon heterotypic E-cadherin ligation.

Importance: The above experiments provide a 'proof of concept' that E-cadherin can participate in transformed cells *in vitro*. These studies challenge the dogma that E-cadherin ligation can only occur in homotypic populations of healthy epithelial cells.

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